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Nucleotide sequence of cDNA encoding the fire ant venom protein Sol i II

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For the first time the cDNA encoding a fire ant venom protein has been sequenced. Oligonucleotides were designed according to the amino acid sequence. The cDNA sequence was obtained by hybridizing these primers to mRNA and enhancement by the PCR technique. Comparison to the amino acid sequence of the venom protein shows a leader sequence 19 amino acids long.

Allergy; Insect venom; cDNA cloning; PCR

1. INTRODUCTION

The red imported fire ant, *Solenopsis invicta*, is an aggressive predator and has been recognized as a serious health hazard [1]. Stings from these ants have become the leading cause of insect venom allergic reactions in the southeastern part of the United States. Four allergenic proteins have been isolated from the venom (Sol i I–IV) with molecular weights between 20,000 and 37,000 daltons [2]. The amino acid sequence of Sol i II has been determined by Hoffman [3] and shows 119 amino acids and a molecular weight of 13,217 daltons. This protein appears to be structurally unrelated to any other protein reported in the PIR and Swiss-Prot databases [3]. This work is the first report of the cDNA sequence of one of the fire ant venom protein allergens, Sol i II.

2. MATERIALS AND METHODS

2.1. *Isolating mRNA*

The isolation of mRNA was carried out using modified standard methods [4] including the use of a high molar concentration of guanidinium and poly(A) selection with oligo(dT) cellulose. Briefly, approximately 3.2 g of fire ants were washed with PBS and homogenized in a buffer containing 6 M guanidine isothiocyanate, 0.05 M Tris-HCl, pH 7.5, 0.01 M Na_2-EDTA, 5% β-mercaptoethanol. Insoluble material was removed by centrifugation and 1/10 volume of *N*-lauroylsarcosine (10%) was added to the supernatant followed by 2 extractions with phenol/chloroform/isoamyl alcohol (25:24:1) and 1 extraction with chloroform. The nucleic acid was precipitated with ethanol, the pellet resuspended in 7.5 ml RNA buffer (140 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris, pH 8.6, 10% Nonidet P-40, 100 mM dithiothreitol, 20 mM vanadyl ribonucleoside complex) and 7.5 ml lysis buffer (200 mM Tris, pH 8.0, 25 mM EDTA, pH 8.0, 300 mM NaCl, 20% SDS) and passed through a syringe fitted with an 23-gauge needle. After 300 μl of proteinase K (20 mg/ml) were added the sample was incubated for 20–30 min at 45°C. The salt concentration was adjusted to 0.5 M NaCl and the lysate was added to 0.5 mg oligo(dT) cellulose, which was equilibrated with binding buffer (50 mM sodium citrate, 500 mM NaCl, 1 mM EDTA, 0.1% *N*-lauroylsarcosine) The sample was incubated for 45 min with gentle agitation at room temperature. After several washings with binding buffer the sample was transferred onto a spin column (Millipore SK1M 525 J8) and washing continued until the OD_{260} was less than 0.05 absorbance. The mRNA was eluted with elution buffer (10 mM Tris, pH 7.6, 1 mM EDTA, pH 8.0, 0.05% SDS) and precipitated with ethanol at −70°C

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2.2. *Construction of oligonucleotide primers for PCR*

Three degenerate oligonucleotides (053, 072 and 073) were designed according to the amino acid sequence. The primer 053 was in anti-sense direction and close to the C-terminal end, whereas 072 and 073 were in the sense orientation and corresponded with the N-terminal end and 34 amino acids inward respectively. In addition, an oligo(dT) primer and an exact match primer in the anti-sense orientation (163) were used. All primers are listed in Table I.

2.3. *PCR amplification*

Polymerase chain reaction (PCR, Perkin-Elmer Cetus, Norwalk, CT),was used to amplify the RNA and to obtain three overlapping cDNA fragments, using the following primer combinations: 053 and 072, oligo(dT) and 073, 163 with 5′ RACE protocol.

The amplification of the middle fragment of Sol i II venom cDNA was based on an adaptation of the 5′ RACE System (Gibco BRL, Gaithersburg, MD). Specifically, 1 μg of poly(A) selected RNA was added to 1.0 μmole of oligonucleotide 053 in a 55 μl volume, and heated to 70°C for 10 min, then chilled on ice. The following solutions were added to the specified final concentration in 1 × Taq buffer II (Perkin-Elmer Cetus): 2 mM dithiothreitol, 0.2 mM each dNTP, 2.0 mM $MgCl_2$. After 2 min at 42°C, 200 units of Superscript RNase H^- (BRL) were added and the reaction incubated for 30 min at 42°C. The mixture was then heated to 55°C, 2 units of RNase H added, and incubated 10 min at 55°C, then chilled on ice. The second oligonucleotide (072) was then added to the reaction mixture, 100 μl of mineral oil was overlaid, and heated to 96°C for 6 min. The temperature was then dropped to 80°C and 5 units of Taq polymerase (Perkin-Elmer Cetus) were added. The mixture was then subjected to 40 cycles of

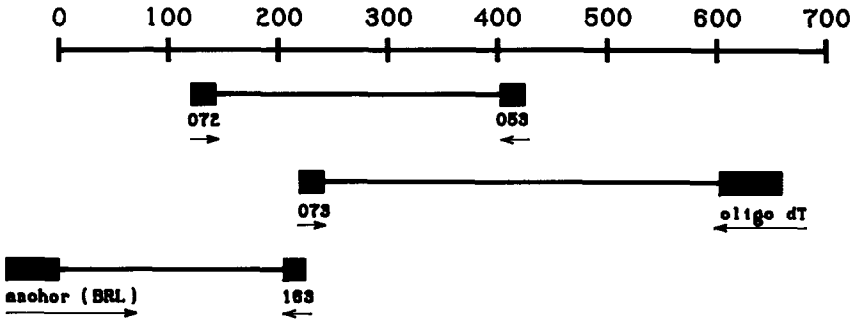

Fig. 1. Strategy of sequencing cDNA by PCR. The graph shows the overlapping fragments and their positions. The blocks on each end indicate the primers.

amplification in an MJ Research Minicycler: 1 min at 94°C, 2 min at 50°C, and 2 min at 72°C.

The 3′ fragment was PCR amplified using the same conditions as above and the primers oligo(dT) [5] and 073.

The 5′ fragment was obtained using primer 053 for the initial cDNA synthesis followed by the gene specific primer 163 using the 5′ RACE System (BRL) according to the manufacturer's protocol.

2.4. *Subcloning*

All three types of Sol i II fragments were analyzed by electrophoresis and the DNA's of positive samples were electroeluted from the agarose gels. The isolated DNA's were digested with restriction endonucleases (RE) which correlated with the RE site incorporated into the original primers The fragments were then ligated into the polylinker site of pUC 19 plasmid. XL1-Blue cells (Stratagene) were transformed using ligation mix, and plated onto L-ampicillin plates containing IPTG and Bluo-Gal (BRL/Life Technologies). Plasmid DNA minipreparations from white bacterial colonies were digested with appropriate RE and analyzed on ethidium bromide agarose gels. DNA's from the corresponding positive plasmid DNA minipreparations were used in sequencing reactions.

2.5. *Double-stranded DNA sequencing*

Recombinant plasmids containing the inserts were denatured in 1 M NaOH for 5 min and 1 pmol of either the pUC/M13 forward or reverse sequencing primer was added together with 0.3 M NaOAc, pH 4.5. After ethanol precipitation the DNA was resuspended in 10 μl Sequenase buffer (US Biochemical). The sequencing reactions then proceeded according to the manufacturer's protocol (Sequenase version 2.0 kit US Biochemical).

3. RESULTS AND DISCUSSION

In order to determine the cDNA sequence of Sol i II venom protein we utilized a PCR amplifying system and standard cloning techniques. We first amplified a cDNA middle fragment using degenerate primers 053 and 072, which were designed according to the known amino acid sequence. The 3′ fragment was amplified with an oligo(dT) and the degenerate primer 073. Finally the 5′ fragment was obtained with primer 053 to synthesize the cDNA followed by the gene specific primer 163 in combination with the anchor primer provided in the 5′ RACE kit (BRL/Life Technologies). The overlapping fragments are illustrated in Fig. 1. For each fragment at least two independent PCR reactions were performed.

The PCR fragments were isolated, ligated into pUC19 and used to transform XL1-Blue bacterial cells (Stratagene). Positive plasmid recombinants were identified by RE digestion and approximate size determination of the DNA inserts. One clone of each independent PCR reaction was then sequenced. The sequences were computer analyzed using the DNASIS software program (National Biosciences). The independent clones of each fragment showed the same sequence and the resulting fragments were then linked together to obtain the entire cDNA sequence. The full cDNA is 605 nucleotides in length (Fig. 2) including 128 nucleotides after the last encoded amino acid up to the beginning of the poly(A) tail. The bracketed nucleotides immediately preceding the pA tail (Fig. 2) indicate the nucleotide sequence found in the longest of the three independent clones. The second and third independent clone were missing either the nine bracketed nucleotides, or the six most pA tail proximal nucleotides within the brackets. These differences may be due to a slight variability in the site of the oligo(dT) primer annealing or to the presence of different alleles in the fire ant population

Table I

List of primers used in PCR

053:	5′-GGCGGATCC(A/G/T)AT(A/G/T)AT(A/G)TTNACNGC(C/T)TT(C/T)TG-3′
072:	5′-GCGGAATTCGA(T/C)AA(T/C)AA(A/G)GA(A/G)(T/C)TNAA(A/G)AT(T/C/A)AT-3′
073:	5′-CTCGAATTCGA(T/C)GTNTGGCA(T/C)TG(T/C)GCNATGGC-3′
163:	5′-ACAGAATTCTACATCGACTCTAGCTAATGG-3′
oligo dT:	5′-AAGGATCCGTCGACATCGATAATACGACTCACTATAGGGATTTTTTTTTTTTTTTT-3′
anchor: primer (BRL)	5′-CUACUACUACUAGGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′

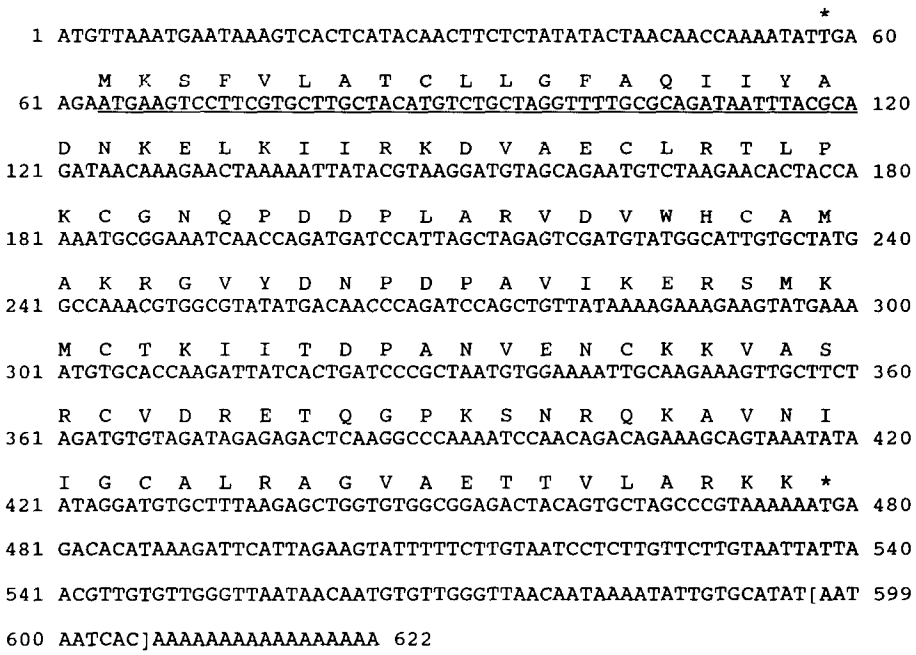

Fig 2. cDNA and translated amino acid sequence for fire ant venom Soli II. The leader sequence is underlined and the asterisks indicate stop codons. The nine nucleotides in brackets at the poly(A) tail represent the deletion area found in different clones.

from which the RNA was made. The inferred amino acid sequence is identical to that previously published [3]. The data also suggest a leader sequence corresponding to 19 amino acids which has not been previously reported because the protein sequence data were obtained from the protein in the venom released by stings and not from the venom within the gland cells.

The cDNA sequence was compared with other nucleotide sequences using the NCBI Blast E-mail search [6]. No significant homology was found with reported sequences.

Sol i II is a major allergen of imported fire ant venom, and most patients show reactivity to it. This is the first report of the successful cloning and sequencing of a fire ant venom protein. With similar methods it should be possible to sequence the cDNA's coding for the other proteins of the venom. Using a baculovirus expression system which utilizes insect cells, it should be possible to express the fire ant venom proteins, hopefully in native conformation. The allergenic activity of these venom proteins is dependent upon native conformation [7,8]. It is possible to obtain imported fire ant venom by either laborious hand milking or by mass electrical stimulation [1], but the venom is expensive and the proteins are contaminated with the alkaloids which comprise about 95% of the venom. Knowledge of the sequence and the ability to express fragments of the molecule that include major T cell epitopes may allow the production of relatively non-allergenic peptides that can be used to down- regulate the production of IgE antibodies [9]. This could eventually lead to immunotherapy with a mixture of T cell epitope peptides, rather than with highly allergenic venom proteins.

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